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ABSORPTION OF MINERALS BY INTESTINAL CELLS

This invention relates to a method for facilitating or increasing the absorption, by mammals, of minerals from the general diet. In particular, this invention relates to a method which involves the administration of an enteral composition containing *Lactobacilli* micro-organisms.

Minerals are key elements in major physiological processes. Calcium is, for example, of vital importance for the formation of bones and teeth, muscle contraction and the synthesis of hormones. Calcium is also an essential secondary messenger in most cell activation phenomena.

Minerals, of which the diet is the primary source, are assimilated by the body by crossing the intestinal mucosa so as to then pass into the blood stream. The degree of assimilation (or of absorption) of minerals by the body in fact depends both on their solubility in the intestinal medium and on the capacity of the intestinal cells to assimilate them and to transfer them into the blood stream (R. Wasserman *et al.*, *In Mineral Absorption in the Monogastric GI Trac*, Advances in Experimental Medicine and Biology, 249, 45-65, Plenum Press, N.Y., 1989).

The location, the efficiency and the mechanisms of calcium absorption all along the intestine have been studied in rats and chickens for many years (Bronner F., *J. Nutr.*, 122, 641-643, 1992; Schachter D., *Am. J. Physiol.*, 196, 357-362, 1959). For obvious ethical and technical reasons, such studies have been limited in man (Hylander E. *et al.*, *Scand. J. Gastroenterol.*, 25, 705, 1990) and only a few *in vitro* studies have been undertaken (Elsherydah A. *et al.*, *Gastroenterology*, 109, 876, 1995; Feher J.J., *Am. J. Physiol.*, 244, C303, 1983; Feher J.J., *Cell Calcium*, 10, 189, 1989).

One of the most widely studied aspects of mineral absorption is the bioavailability of the minerals depending on the composition of the daily diet (Bronner F., *J. Nutr.*, 123, 797, 1993). However, many minerals which are highly bioavailable are also instable and are unsuitable for use in the diet. Further, merely supplementing the diet with greater amounts of minerals often has a negative effect on the organo-leptic properties of the diet.

A possible solution to the problem is to facilitate or improve the absorption of minerals from the diet. However there have been few studies on methods of facilitating or increasing the absorption of minerals from the diet and the results have not been consistent.

5 More recently, Yaeshima *et al.* have also shown an increase in the absorption of calcium in rats from a diet of calcium-fortified whey when a combination of oligosaccharides and *Bifidobacteria* is consumed (XP002052237: Bulletin of the International Dairy Fermentation, No. 313, 1996).

Therefore there remains a need for a means of facilitating or increasing the absorption of minerals present in the diet.

It has been surprisingly found, by use of an *in vitro* model, that *lactobacilli* are able to directly facilitate or improve the absorption of minerals, especially calcium, by human intestinal cells. Without wishing to be bound by theory, this is thought to be linked to induction of acidification of the microenvironment around the intestinal cells and the bacteria in contact with the intestinal cells. Both the bacteria and the intestinal cells may participate in the induction of acidification. This localized acidification might thus play an active role in the solubilization of minerals, and therefore in the capacity of the body to assimilate them.

B Embodiments of the invention are now described, by way of example only, with reference to the drawings in which:

35 Figure 2 represents the influence of about 6.7×10^7 cfu/ml of various strains of *lactobacilli* on the absorption of calcium by Caco-2 intestinal cells;

Figure 3 represents the influence of about 3.4×10^8 cfu/ml of various strains of *lactobacilli* on the absorption of calcium by Caco-2 intestinal cells.

The invention relates to the enteral administration of a nutritional composition which contains *lactobacilli* to facilitate or improve the absorption of minerals present in a daily diet. Examples of minerals are calcium, magnesium, iron and/or zinc. The ingestion of *lactobacilli* increases the bioavailability of the minerals, that is to say makes the minerals, which are often not very soluble in the intestine, more accessible to the intestinal cells.

10 *Sub C1* Any food-grade, *lactobacilli* strain which may be used. For example, the following *lactobacilli* may be used: *Lactobacillus acidophilus*, *Lactobacillus crispatus*, *Lactobacillus amylovorus*, *Lactobacillus gallinarum*, *Lactobacillus gasseri* and *Lactobacillus johnsonii*; *Lactobacillus paracasei*; *Lactobacillus reuterii*; *Lactobacillus brevis*; *Lactobacillus fermentum*; *Lactobacillus plantarum*; *Lactobacillus casei* especially *L. casei* subsp. *casei* and *L. casei* subsp. *rhannosus*; *Lactobacillus delbrückii* especially *L. delbrückii* subsp. *lactis*, *L. delbrückii* subsp. *helveticus* and *L. delbrückii* subsp. *bulgaricus*; and *Leuconostoc mesenteroides* especially *L. mesenteroides* subsp. *cremoris*, for example (Bergey's Manual of Systematic Bacteriology, vol. 2, 1986; Fujisawa et al., Int. Syst. Bact, 42, 487-491, 1992).

20 The *lactobacilli* may be capable of adhering to intestinal cells but need not be. However, the *lactobacilli* are preferably such that at least 50 bacteria, in particular at least 80 bacteria, are capable of adhering *in vitro* to 100 intestinal cells. To select such an adherent type of bacteria, a culture of bacteria may be spread on a confluent culture of an immortalized line of epithelial cells of the intestine (EP 0802257), the confluent culture washed, and the number of bacteria adhering to the villusities of the line measured.

30 Probiotic *lactobacilli* are of particular interest. Some strains are in fact capable of adhering to human intestinal cells, of excluding pathogenic bacteria which are on human intestinal cells, and/or of acting on the human immune system by allowing it to react more strongly to external aggression (immunomodulation capacity), for example by increasing the phagocytosis capacity of the granulocytes derived from human blood (J. of Dairy Science, 78, 491-197, 1995; immunomodulation capacity of the La-1 strain which was deposited by Nestec SA with the treaty of Budapest in the Collection Nationale de Culture de Microorganisme (CNCM), 25 rue docteur Roux, 75724 Paris,

30 June 1992, where it was attributed the deposit number CNCM I-1225). This strain is described in EP 0577904

By way of example, it is possible to use the probiotic strain *Lactobacillus acidophilus* CNCM I-1225. This strain was recently reclassified among the *Lactobacillus johnsonii* bacteria, subsequent to the new taxonomy, proposed by Fujisawa *et al.*, which is now authoritative in the field of taxonomy of acidophilic lactobacilli (Int. J. Syst. Bact., 42, 487-791, 1992). Other probiotic bacteria are also available, such as those described in EP0199535 (Gorbach *et al.*), US5296221 (Mitsuoka *et al.*), US556785 (Institut Pasteur) or US5591428 (Probi AB), for example.

The nutritional compositions preferably comprise a sufficient quantity of live *lactobacilli* for a facilitated absorption of minerals by the intestinal cells, for example at least 10^6 cfu/ml, in particular 10^7 - 10^{11} cfu/ml, preferably 10^8 - 10^{11} cfu/ml ("cfu" means "colony forming unit").

The nutritional composition may also contain other bacteria as desired; for example other probiotic bacteria.

The nutritional composition may also include a suitable protein source; for example an animal or plant protein source. Suitable protein sources are milk proteins, soy proteins, rice proteins, wheat proteins, sorghum proteins, and the like. The proteins may be in intact or hydrolyzed form.

The nutritional composition may also include a suitable carbohydrate source; for example sucrose, fructose, glucose, maltodextrin, and the like.

The nutritional composition may also include a suitable lipid source; for example a suitable animal or plant lipid source. Suitable lipid sources include milk fats, sunflower oil, rapeseed oil, olive oil, safflower oil, and the like.

The nutritional composition may also be fortified with minerals and vitamins. It is especially preferred to fortify the nutritional composition with calcium.

The nutritional compositions may be prepared in the form of food compositions intended for human or animal consumption. Suitable food compositions may be provided in the form of liquids, powders, and solids.

The nutritional composition may be fermented to obtain a sufficient quantity of *lactobacilli*. Fermented compositions based on milk are thus particularly suitable. The term milk applies not only to animal milks but also to what is commonly called a vegetable milk, that is to say an extract of treated or untreated plant materials such as legumes (soya, chick pea, lentil and the like) or

oilseeds (rape, soya, sesame, cotton and the like), which extract contains proteins in solution or in colloidal suspension, which are coagulable by chemical action, by acid fermentation and/or by heat. It has been possible to subject these vegetable milks to heat treatments similar to those for animal milks. It has also been possible to subject them to treatments which are specific to them, such as decolorization, deodorization, and treatments for suppressing undesirable tastes. Finally, the word milk also designates mixtures of animal milks and of plant milks.

It is also possible to add, mix or coat the nutritional composition, during its preparation, with an appropriate quantity of a culture of *lactobacilli* in liquid, concentrated, dry or encapsulated form, according to need.

It has been found that the microencapsulation of the *lactobacilli* has therapeutic advantages. First, microencapsulation significantly increases the survival of the *lactobacilli* and therefore the number of live *lactobacilli* which arrive in the intestine. Even more importantly, the *lactobacilli* are gradually released into the intestine, which permits prolonged action of the *lactobacilli* on the absorption of minerals by the intestinal cells.

Preferably, to encapsulate *lactobacilli*, the *lactobacilli* are freeze-dried or spray-dried (EP0818529), and they are incorporated into a gel consisting, for example, of a solidified fatty acid, a sodium alginate, polymerized hydroxypropylmethylcellulose or polymerized polyvinylpyrrolidone. To this effect, the teaching given in FR2,443,247 is incorporated by reference.

The nutritional compositions need not contain carbohydrates necessary for active fermentation by *lactobacilli* in the intestinal medium. On the contrary, the facilitated absorption of minerals is independent of the fermentative activity of the *lactobacilli*, but rather appears to result from the direct contact between the *lactobacilli* and the intestinal cells. This is thought to induce acidification of the microenvironment and therefore a better solubilization of the minerals.

However, it may be desirable to provide for renewal or specific multiplication of the *lactobacilli* in the intestinal medium so as to prolong the effect of facilitated absorption of the minerals. This may be achieved by adding fibres which facilitate the specific multiplication of *lactobacilli* in the intestinal medium to the nutritional composition. These fibres are soluble and fermentable.

These fibres may be selected from, for example, plant pectins, chito-, fructo-, gentio-, galacto-, isomalto-, manno- or xylo-oligosaccharides or

oligosaccharides from soya. for example (Playne et al., Bulletin of the IDF 313, Group B42, Annual Session of September 95, Vienna).

The preferred pectins are polymers of α -1,4-D-galacturonic acid having a molecular weight of the order of 10 to 400 kDa, which can be purified from carrots or tomatoes, for example (JP60164432). The preferred galacto-oligosaccharides comprise a saccharide portion consisting of 2 to 5 repeating units of structure $[-\alpha\text{-D-Glu-(1}\rightarrow\text{4)-}\beta\text{-D-Gal-(1}\rightarrow\text{6)-}]$ (Yakult Honsa Co., Japan). The preferred fructo-oligosaccharides are inulin-oligofructoses extracted from chicory which may comprise, for example, 1-9 repeating units of structure $[-\beta\text{-D-Fru-(1}\rightarrow\text{2)-}\beta\text{-D-Fru-(1}\rightarrow\text{2)-}]$ (WO94/12541; Raffinerie Tirlemontoise S.A., Belgium), or oligosaccharides synthesized from sucrose units which may comprise, for example, a saccharide portion consisting of 2 to 9 repeating units of structure $[-\alpha\text{-D-Glu-(1}\rightarrow\text{2)-}\beta\text{-D-Fru-(1}\rightarrow\text{2)-}]$ (Meiji Seika Kashi Co., Japan). The preferred malto-oligosaccharides comprise a saccharide portion consisting of 2 to 7 repeating units of structure $[-\alpha\text{-D-Gal-(1}\rightarrow\text{4)-}]$ (Nihon Shokuhin Kako Co., Japan). The preferred isomaltoses comprise a saccharide portion consisting of 2 to 6 repeating units of structure $[-\alpha\text{-D-Glu-(1}\rightarrow\text{6)-}]$ (Showa Sangyo Co., Japan). The preferred gentio-oligosaccharides comprise a saccharide portion consisting of 2 to 5 repeating units of structure $[-\beta\text{-D-Glu-(1}\rightarrow\text{6)-}]$ (Nihon Shokuhin Kako Co., Japan). Finally, the preferred xylo-oligosaccharides comprise a saccharide portion consisting of 2 to 9 repeating units of structure $[-\beta\text{-xyl-(1}\rightarrow\text{4)-}]$ (Suntory Co., Japan), for example.

The quantity of fibres in the nutritional composition depends on their capacity to promote the development of *lactobacilli*. As a general rule, the nutritional composition may contain from 1 to 50% of such fibres (by weight relative to the dry matter). The concentration of *lactobacilli* may be at least 10^3 CFU of *lactobacilli* per g of fibres, preferably 10^4 to 10^7 CFU/g of fibres.

Another advantage provided by the fibres consists in the fact that the intestinal transit is retarded by the fibres. This is particularly the case if the quantity of fibres is large, that is to say of the order of 20-50% relative to the weight of the composition. The *lactobacilli* being gradually eliminated by the action of the intestinal transit, it is possible, in this manner, to prolong the beneficial action of the *lactobacilli* on the absorption of minerals by the intestine.

The nutritional compositions may be in the form of any suitable enterally administered food. For example, the nutritional composition may take the form of a fermented milk (EP0577904), an infant (EP0827697), a fromage frais

(PCT/EP97/06947), a ripened cheese, an ice cream (WO 98/09535), a biscuit filled with a cream (EP704164; EP666031), a dry sausage and/or a pâté (EP689769).

5 The nutritional compositions may also be in a form suitable for people who cannot tolerate dairy products. These nutritional compositions will not contain allergenic milk derivatives. For example, for children who are allergic to milk proteins, the nutritional composition may be formulated to contain hypoallergenic milk derivatives. These milk derivatives may be in accordance with European directive 96/4/EC which states that in a hypoallergenic milk, the
10 allergenic proteins should be immunologically at least 100 times less detectable than in a nonhydrolysed milk (Off. J. Europ. Comm., NoL49/12, annex point 5.a. 1996; Fritsché *et al.*, Int. Arch. Aller. and Appl. Imm., 93, 289-293, 1990).

The nutritional compositions are particularly suitable for the treatment or prophylaxis of people having mineral deficiencies, or to compensate for
15 physiological deficiencies due to a diet low in minerals, or to satisfy major physiological requirements for minerals in children, pregnant women, women who are breastfeeding and the elderly.

This invention is now further described by means of specific examples. The percentages are given by weight unless otherwise indicated. These examples
20 are given by way of illustration only and do not in any manner constitute a limitation of the invention.

Example 1

25 - Materials: $^{45}\text{CaCl}_2$ is obtained from Amersham, Lucifer yellow from Sigma, collagen I from Centrix Pharmaceuticals, PBS, HEPES and the components of the cell culture medium from Gibco, and the culture supports from Falcon.

- Cell culture: the human cell line Caco-2, isolated from a colon adenocarcinoma, is obtained from American Type Culture Collection (passage 41). The cells are placed in culture in an amount of 4×10^4 cells/cm² in DMEM
30 containing 4.5 g/l of glucose, 20% heat-inactivated foetal calf serum, 1 mg/ml of fungizone, 100 U/ml of penicillin/streptomycin, 200 µg/ml of gentamycin and 1% of nonessential amino acids. The cells are regularly trypsinized and placed in culture again at 1:20. The cells used in the calcium transport experiments are placed in culture at 1×10^5 cells/cm² in permeable inserts previously coated with a
35 layer of collagen I at 50 µg/ml. In all cases, the cells are maintained in a 10% CO₂/90% air incubator at 37°C, and the medium is replaced every two days.

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Lactobacillus bulgaricus Lfi5, YL8; *Lactobacillus paracasei* ST11;
Lactobacillus gasseri LGA7; *Lactobacillus reuteri* LR7 and *Streptococcus*
thermophilus Sfi20, YS4 (Nestec collection, Lausanne, Switzerland) are placed
in culture under anaerobic conditions in MRS broth for *Lactobacillus* or M17 for
5 *Streptococcus* for two times 24 h, washed in PBS and resuspended in carrier
buffer before being introduced into the top compartment of the inserts. The
Caco-2:bacteria ratio is then about 1:100 according to the tests (6.7×10^7 or
 3.4×10^8 cfu/ml in the top compartment of the inserts, for the tests presented in
Figures 2 and 3). The absorption of calcium is evaluated according to the
10 protocol mentioned above.

- Results of the basal transport of calcium: a calcium gradient was established in
the inserts by introducing 2.5 mM CaCl_2 into the bottom compartment, which
corresponds to the normal human plasma concentration, and arbitrarily 10 mM
 CaCl_2 into the top compartment, which would correspond to the calcium content
15 of a food diet. As shown by the results of a representative experiment illustrated
by Figure 1, the basal absorption of calcium by the Caco-2 cells increases with
time to reach up to 600 nmol/insert, comprising about 3×10^6 cells, after 4 h. As a
check for the integrity of the cellular lawn during the experiment, the diffusion of
Lucifer yellow was measured and proved to be less than 2%.

- Measurement of the influence of lactobacilli: in Figures 2 and 3, the absorption
of calcium by the Caco-2 cells is increased significantly in the presence of the
adherent *Lactobacillus johnsonii* strains La1 and La22, in the presence of the
non-adherent La10 and La18 *Lactobacillus acidophilus* strains, and in the
presence of the *L. paracasei* (ST11), *L. gasseri* (LGA7) and *L. reuterii* (LR7)
25 strains.

The capacity of the bacteria to adhere to the intestinal cells therefore does
not appear to correlate directly with their capacity to increase the absorption of
calcium by these same cells. In all these experiments, the diffusion of Lucifer
yellow is modulated in a similar manner but remains negligible.

30 A decrease in pH in the top compartment of the inserts is also observed
when the Caco-2 cells are in the presence of *lactobacilli*, regardless of the strain,
except with the Sfi20 strain (Table 1). There is therefore no correlation between
the increase in the absorption of calcium and this decrease in pH. However
certain bacterial strains capable of increasing the absorption of calcium are not
35 capable of acidifying the experimental medium in the absence of Caco-2. This
means that the acidification in the presence of Caco-2 and of bacteria requires a

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collaboration between the two types of organisms and could be due to the Caco-2 cells.

Table 1: Influence of *lactobacilli* on the pH of the experimental medium in the absence or in the presence of Caco-2 cells

| Bacteria | Number of tests | pH without Caco-2 | pH with Caco-2 |
|----------|-----------------|-------------------|----------------|
| None | 4 | 7 +/- 0 | 7 +/- 0 |
| La1 | 3 | 6.75 +/- 0.3 | 3.75 +/- 0.3 |
| La10 | 3 | 4.65 +/- 0.3 | 4.15 +/- 0.3 |
| La17 | 2 | 7 +/- 0 | 3.5 +/- 0.7 |
| La18 | 2 | 7 +/- 0 | 3.5 +/- 0.5 |
| La22 | 2 | 7 +/- 0 | 3.25 +/- 0.35 |
| La29 | 2 | 4.25 +/- 0.35 | 3.5 +/- 0 |
| La31 | 2 | 7 +/- 0 | 3.75 +/- 0.35 |
| Sfi20 | 1 | 7 | 7 |
| YS4 | 1 | 5 | 4 |
| Lfi5 | 1 | 4 | 3 |
| YL8 | 1 | 4 | 3 |

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Example 2

Tests similar to those carried out in Example 1 were carried out to determine the influence of *lactobacilli* on the absorption of calcium by the intestinal cells in the presence of labelled inulin (^3H -inulin, Amersham; tracer prebiotic fibre). The results confirm that *lactobacilli* increase *in vitro* the absorption of minerals by the intestinal cells.

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Example 3

Tests similar to those carried out in Example 1 were carried out in order to determine the influence of *lactobacilli* on the absorption of magnesium, iron and zinc by the intestinal cells. The results confirm that *lactobacilli* increase *in vitro* the absorption of minerals by the intestinal cells.

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Example 4 Encapsulation of lactic acid bacteria

In a 100 l tank, 80 l of culture medium having the following composition, in %, are prepared:

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| Yeast extract | 0.25% |
| Trypticase | 1.00% |
| Phytone | 0.50% |
| Glucose | 1.50% |
| L-cysteine HCl | 0.05% |
| K ₂ HPO ₄ | 0.25% |
| ZnSO ₄ | 0.025% |
| FeCl ₃ | Trace |
| Water | Balance to 100% |

Inoculation is carried out with 1 l of a 20 h culture of *Lactobacillus johnsonii* La1 (CNCM I-1225). The medium is incubated for 12 h at 30°C. The culture broth is centrifuged and 240 g of cells are recovered. They are diluted in 250 ml of skimmed milk supplemented with 7% lactose. The mixture is frozen using liquid nitrogen. The freeze-drying is performed at 40°C overnight. A 5% dispersion of the powder obtained is prepared in hydrogenated vegetable fat having a melting point of 42°C and liquefied at 45°C. The dispersion is injected at 45°C under a pressure of 4 bar, at the same time as liquid nitrogen, in an amount of 1 part of dispersion for 5 parts of nitrogen, at the top of a vertical cylinder 1.5 m in diameter and 10 m high. A container is placed at the bottom of the cylinder, which contains liquid nitrogen in which the microbeads containing the bacteria whose diameter varies between 0.1 and 0.5 m are collected. The microbeads are then placed in a fluidized bed and an alcoholic solution containing 8% zein is sprayed over the bed, in a quantity such that the zein layer formed around the microbeads represents 5% of their weight.

The microbeads are then incorporated into a food composition intended to facilitate the absorption of minerals by the intestinal cells.

Example 5

A concentrated base for ice cream is prepared by mixing at 60-65°C for 20 min about 11% of lactic fat, 8.8% of milk solids (solids-not-fat), 25% sucrose, 5% of glucose syrup and 0.6% of Emulstab® SE30. The base is homogenized at 72-75°C and at 210 bar (2 stages at 210/50 bar), it is pasteurized at 85°C for 22 sec (APV pasteurizer, France, Evreux, 400 l/h), it is cooled to 4°C and 40% of

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milk acidified by *Lactobacillus johnsonii* La-1 (5×10^8 cfu/ml) and *Bifidobacterium longum* Bi16 (3×10^8 cfu/ml) strains is added thereto. The composition of this concentrated base is presented in the table below.

| Ingredients | Composition (kg) | Fat (%) | Solids-not-fat (%) | Sucrose (%) | Dry extract (%) |
|------------------------------------|------------------|---------|--------------------|-------------|-----------------|
| Cream (35%) | 31.43 | 11.00 | 1.57 | | 12.57 |
| Skimmed milk powder | 7.60 | | 7.30 | | 7.30 |
| Sucrose | 36.77 | | | 25.00 | 25.00 |
| Glucose syrup | 5.27 | | | | 5.00 |
| Emulstab [®] SE30 | 0.67 | | | | 0.63 |
| Water | 18.26 | | | | |
| Total: cream base | 100.00 | 11.00 | 8.87 | 25.00 | 50.50 |
| Cream base (60%) | 60.00 | 6.60 | 5.32 | 15.00 | 30.30 |
| Acidified milk (40%) | 40.00 | 1.40 | 4.68 | - | 6.08 |
| Total: cream base + acidified milk | 100.00 | 8.00 | 10.00 | 15.00 | 36.38 |

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After maturation of the cream for 12 h at 5°C, it is frozen to an overrun of 95% by volume (Crepaco freezer, France, Evreux; 160 l of product/h).

10 A wafer dough is prepared which contains 10% fructo-oligosaccharide Raftilose[®] L30 (Raffinerie Tirlemontoise S.A., BE), according to the recipe reproduced in the table below. After baking, the wafer is conventionally formed into a cone. After cooling, the inside of the cones is spray-coated with a fatty film and then the cones are filled with the whipped ice cream described above. For an

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11.5 g wafer cone, 130 ml of whipped ice cream (about 65 g) and 5 g of chocolate (spraying over the cream) are thus used.

| Ingredient | Weight (g) | Supplier |
|----------------------------|------------|--------------------------------------|
| Ordinary wheat flour 55 | 52 | Raffinerie Tirlemontoise S.A., BE |
| Starch | 0.2 | |
| Fructo-oligosaccharide | 10 | |
| Raftilose [®] L30 | | |
| Sugar | 27.8 | |
| Fat | 8 | |
| Emulsifier | 1.5 | |
| Salt | 0.5 | |
| Total: wafer recipe | 100 | |

- 5 1.1 g of fibres and about 10^8 cfu/g of *lactobacilli* are thus provided per ice cream cornet. The fibres, by promoting the specific development of *lactobacilli* in the intestinal tract, thus promote the assimilation of minerals.

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